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The Role of Receptor Dimerization Domain Residues in Growth Hormone Signaling*

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While there is a considerable amount of evidence that signal transduction by the growth hormone (GH) receptor requires receptor homodimerization, there has been no systematic study of the role of receptor dimerization domain residues in this process. In conjunction with the distances derived from the crystal structure of the hGH-hGH receptor (extracellular domain) complex, we have used a luciferase-based c-*fos* promoter reporter assay in transiently transfected Chinese hamster ovary (CHO) cells, and stable receptor expressing CHO cell populations to define the dimerization domain residues needed for effective signaling. In addition to alanine substitution, we have used both aspartate and lysine substitutions to allow us to provide evidence for proximity relations through charge complementation. Introduced cysteine substitutions were also used, but unlike the erythropoietin receptor, these were unable to generate constitutively active receptor. We conclude that serine 145, histidine 150, aspartate 152, tyrosine 200, and serine 201, but not leucine 146 or threonine 147 are required for effective signal transduction through the dimerization domain. This information may be valuable in designing small molecule antagonists of GH and other cytokines that block dimerization by binding to the dimerization domain.

Dimerization of receptors induced by ligand binding is a common means of triggering signal transduction in mammalian cells (1). In a number of members of the tyrosine kinase receptor family (*e.g.* platelet-derived growth factor and epidermal growth factor), two ligand molecules bind to two receptors to form a L_2R_2 complex, which is followed by activation of the receptor tyrosine kinase. By contrast, in the cytokine receptor superfamily, one ligand molecule promotes dimerization of heterologous receptor subunits or dimerization of two identical subunits (2, 3). Currently, the best characterized example of ligand-induced receptor dimerization in the cytokine receptor superfamily is the GH receptor (GHR).[1] The crystal structure of the ligand-bound complex (4) shows that hGH binds to two identical receptor (hGHBP) subunits to induce receptor dimerization, which is aided by a dimerization domain in the C-terminal β sandwich (domain 2). Eight residues were identified as being involved in this dimerization domain, and the relationship of these is summarized in Table I. The crystal structure verified previous studies based on several techniques, which demonstrated a 1:2 stoichiometry of hormone to receptor (5). GHR dimerization occurs sequentially, *i.e.* hGH binds to the first hGHR through "site 1" of hGH and subsequently binds to the second hGHR through "site 2" of hGH (5). Cunningham *et al.* (5) proposed that receptor dimerization results in signal transduction, and this proposal was verified by studies from the same group with GH mutants and GH receptor monoclonal antibodies using a proliferation assay for FDC-P1 cells expressing a chimeric hGH-mouse granulocyte colony-stimulating factor receptor (6). A study of GH-induced tyrosine phosphorylation of cellular proteins also showed that tyrosine phosphorylation was inhibited by treating IM-9 cells with a GH mutant that prevents receptor dimerization (7). Furthermore, a mathematical model for the quantitative evaluation of the bell-shaped dose-response curves associated with sequential receptor dimerization and the use of the nondimerizing hGH mutant (G120R)[2] indicated that GHR dimerization is essential for GH stimulation of both lipogenesis in primary rat adipocytes and receptor down-regulation in cultured human IM-9 lymphocytes (8). In support of these studies, a single amino acid substitution (D152H) in the dimerization region of the human GHR identified in a Laron dwarf abolished receptor dimerization (9). GHR dimerization is thought to activate Jak2 kinase, which then phosphorylates both the receptor and downstream signaling components such as the Stats, IRS-1 and Shc (10). Currently, no systematic study of the importance of receptor dimerization domain residues in signal transduction has been carried out by biological function assay. In addition, it is not proven that those residues involved in dimerization in the crystal structure indeed represent residues interacting *in situ*. Accordingly, eight residues in the putative dimerization region of RGHR (based on the crystal structure of hGHBP; Ref. 4) were converted to alanine, to cysteine, to lysine, and to aspartate individually or in combination, and the importance of these residues in signal transduction was examined using a c-*fos* promoter luciferase assay (11) which has been useful in defining structural components of the receptor needed for signaling (12).

MATERIALS AND METHODS

Constructs

Receptor Dimerization Domain Mutants—A rabbit GHR (RGHR) expression plasmid (pECE-RGHR) was constructed by inserting rabbit growth hormone receptor (RGHR) cDNA (13) into the SV40-based mammalian expression vector pECE at *Kpn*I and *Xba*I sites. All RGHR mutants were inserted into the mammalian expression vector pECE as

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[1] The abbreviations used are: GHR, growth hormone receptor; GH, growth hormone; hGH, human growth hormone; RGHR, rabbit growth hormone receptor; GHBP, growth hormone binding protein (extracellular domain of GHR); bp, base pair(s); FCS, fetal calf serum; CHO, Chinese hamster ovary; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate.

[2] Mutants are designated by the wild type residue followed by its position and the introduced mutation in single-letter code.

for wild type pECE-RGHR. The following mutations were made by oligonucleotide-mediated mutagenesis using the Altered Sites *in vitro* mutagenesis system (Promega) with pSELECT and approximately 30 bp of oligonucleotides incorporating 1–3 altered bases, synthesized on a Milligen/Biosearch 7500 DNA synthesizer, and purified by polyacrylamide gel electrophoresis. Multiple alanine substituted mutants were made with the following oligonucleotides: 5′-G ACT TTA CTG GCT GTT GCC GCA GCC GGG ATT GCT GCA GCT ATT CAA GTG C-3′ for A143/A145/A146/A147/A150/A152 (6 Ala); 5′-CT GTA TTG TCG ACA TCA GTT CCT GTG GCC GCG TTA AG-3′ for A200/A201; 5′-CCT GTA TTG TCG ACA TCA GTT CCT GTG TAC GCG TTA-3′ for S201A; 5′-GTT AGC TTA ACC GGG ATT GCT GCA GCT ATT CAA G-3′ for A150/A152. Eight Ala, 4 Ala, and 3 Ala mutants were made by combining these primers, to give the particular Ala substitutions given below.

pECE-RGHR-8A—In this construct, 8 residues in the extracellular region identified in the crystal structure as interacting across the dimerization domain (asparagine 143, serine 145, leucine 146, threonine 147, histidine 150, aspartate 152, tyrosine 200, and serine 201) were converted to alanine.

pECE-RGHR-6A—In this construct, 6 residues (asparagine 143, serine 145, leucine 146, threonine 147, histidine 150, and aspartate 152) were converted to alanine.

pECE-RGHR-4A—For this construct, 4 residues (histidine 150, aspartate 152, tyrosine 200, and serine 201) were converted to alanine.

pECE-RGHR-3A—Here, 3 residues (histidine 150, aspartate 152, and serine 201) were converted to alanine.

pECE-RGHR-Y200,S201A (RGHR-200,201A)—In this construct, 2 residues (tyrosine 200 and serine 201) were converted to alanine.

Other Constructs—Other constructs created with the Altered Sites system were: pECE-RGHR-S201A, pECE-RGHR-S145C, pECE-RGHR-D152C, pECE-RGHR-Y200C, pECE-RGHR-S201C, pECE-RGHR-152,200C, pECE-RGHR-L146D, pECE-RGHR-H150D, pECE-RGHR-Y200D, pECE-RGHR-S201D, pECE-RGHR-N143K, pECE-RGHR-S145K, pECE-RGHR-L146K, pECE-RGHR-T147K, pECE-RGHR-H150K, pECE-RGHR-D152K, pECE-RGHR-S201K, pECE-RGHR-H150K, D152K(RGHR-150, 152K), and pECE-RGHR-Y200,S201K (RGHR-200,201K). All constructs were verified by double-strand dideoxy sequencing using the Pharmacia T7 kit.

c-fos Luciferase Reporter Construct—Plasmid pfos 396Δ219−81 was constructed by inserting the fragment of the human c-*fos* promoter from −396 to +42 into pGL.Basic after deleting the 138 bp from −219 to −81 (11).

Cell Culture

Transient Transfection of CHO K1 Cells—This was undertaken as for Chen *et al.* (11). Briefly, CHO K1 cells maintained in 2 ml of Ham's F-12 medium containing 5% FCS were dispensed into six-well plates at 2.2×10^5 cells/well and left for 20–24 h. After aspiration of the medium, 160 μl of transfection reagent-DNA mixture containing 1 μg of receptor mutant expression plasmid, 2 μg of reporter gene, and 20 μl of DOTAP in HBS (20 mM Hepes, 150 mM NaCl, pH 7.4) was added to each well and mixed gently for 30 s. Three ml of Ham's F-12 medium containing 0.5% FCS was then added to each well. After incubation for 40 h, transfected cells were treated with 200 ng/ml rhGH. Negative control cells were treated with the same volume of serum-free Ham's F-12 medium (medium control). After further incubation for 5.5 h, the cells were harvested for luciferase assay according to Promega's instructions, and normalized to protein content. Constructs were transfected in triplicate for each experiment, and all experiments were repeated a minimum of three times. Transfection controls were as described in Ref. 11, and always included wild type receptor.

Stable Transfection of CHO K1 Cells—CHO K1 cells maintained in 2 ml of Ham's F-12 medium containing 5% FCS were dispensed into six-well plates at 2.2×10^5 cells/well and left for 20–24 h. After aspiration of the medium, 160 μl of transfection reagent-DNA mixture containing 2.7 μg of wild type or mutant RGHR expression plasmid, 0.3 μg of Neo^R expression plasmid, and 20 μl of DOTAP in HBS (20 mM Hepes, 150 mM NaCl, pH 7.4) was added to each well and mixed gently for 30 s. Two ml of Ham's F-12 medium containing 0.5% FCS was then added to each well. After incubation for 16 h, the culture medium was changed to 3 ml of Ham's F-12 medium containing 10% FCS, and the cells were incubated for an additional 48–72 h. Stable transfectants were selected by including G418 in the cell culture medium at 300 ng/ml until the surviving cells reached clonal confluence. The surviving clones were then selected and cultured. An aliquot of each clone was stored frozen, while the reminder of the cells were used for analyses.

Hormone Binding Analysis

Hormone binding constants for cell surface receptor mutants were determined by Scatchard assay using recombinant human GH (a gift from Genentech Inc., South San Francisco, CA) with receptor mutants stably expressed as populations in CHO-K1 cells, since it was not possible to measure receptor transiently expressed, as in the luciferase assay. GH was radiolabeled by the IODOGEN method (14) following by separation on a Sephadex G100 column to give a specific activity range of 100–150 μCi/μg. For assays, 2×10^5 cpm iodinated hormone (less than 1 week old) and eight concentrations (0, 3, 10, 30, 50, 100, 1000, and 10,000 ng/ml) of unlabeled competitor in triplicate were added to cultured cells transfected with wild type or mutant GH receptor. CHO K1 cells stably expressing wild type or mutant RGHR were grown to confluence in six-well plates. Medium was then changed to 25 mM Tris-HCl (pH 7.5), 130 mM NaCl, 0.1% BSA, and 20 mM MgCl, and the assay was carried out essentially as in Ref. 15. After incubation at 10 °C for 16 h, the CHO K1 cells were washed with ice-cold phosphate-buffered saline and harvested by addition of 1 ml of 0.1 M NaOH and incubation for 15 min at room temperature. At 10 °C, internalization is minimal, and the cell membrane is in a highly ordered, gel phase (27). Cell surface-associated counts were quantified using an LKB 1275 γ counter. The resulting data were analyzed and the binding constants calculated using the McPherson LIGAND program (Elsevier Biosoft). Scatchard plots are linear below 50 pM receptor concentration for a dimerizing GH receptor (8), and all estimations herein gave linear Scatchard plots and receptor concentrations below 30 pM.

RESULTS

Alanine Substitutions—Alanine substitution (scanning) is minimally perturbing for secondary and tertiary structure and is generally regarded as the best means of selective removal of interactive residues involved in salt bridges and hydrogen bonds (16, 17). To confirm that alanine-substituted RGHRs were expressed correctly, and to define any impairment of ligand binding, Scatchard assays were performed with stable CHO K1 transfectants. These showed that pECE-RGHR-8A, pECE-RGHR-6A, and pECE-RGHR-S201A were expressed with wild type RGHR affinity and at comparable levels, although pECE-RGHR-8A was expressed at only 28% of wild type number (Table II). These data indicate that alanine mutation in the dimerization domain has no effect on binding affinity of RGHR, and that receptor was expressed at an acceptable level. Fig. 1*A* shows that in the transient cotransfection assay, induction of luciferase activity by GH with pECE-RGHR-8A, pECE-RGHR-6A, pECE-RGHR-4A, pECE-RGHR-3A, and pECE-RGHR-200,201A was less than 25% of wild type pECE-RGHR, and for pECE-RGHR-S201A was 67 ± 4% that of wild type. These results indicate that the residues in the dimerization region observed from crystal structure are indeed involved in signal transduction, particularly Tyr-200 and His-150/Asp-152. Moreover, although the alanine mutants pECE-RGHR-8A, pECE-RGHR-6A, pECE-RGHR-4A, and pECE-RGHR-3A exhibited reduced induction of luciferase activity by GH, they exhibited significantly higher (over 2-fold) basal activity than wild type RGHR (Fig. 1*B*). Therefore, it is possible that these alanine mutants are actually facilitating dimerization through hydrophobic interaction.

Cysteine Substitutions—The homologous erythropoietin receptor is able to exhibit constitutive activation through disulfide bond-initiated homodimerization in the R129C erythroleukemia mutation (18), and also in E132/133C putative dimerization domain mutants (19). To investigate whether an analogous constitutive activation of the GH receptor could be obtained, we substituted four dimerization domain residues (Ser-145, Asp-152, Tyr-200, and Ser-201) with cysteine, and we also created a double cysteine mutant (pECE-RGHR-D152C,Y200C). As indicated in Table I, the latter two residues appear to be hydrogen-bonded in the crystal structure. These mutants were found to be expressed with essentially wild type affinity in CHO-K1 stable populations, and at levels from 76 to

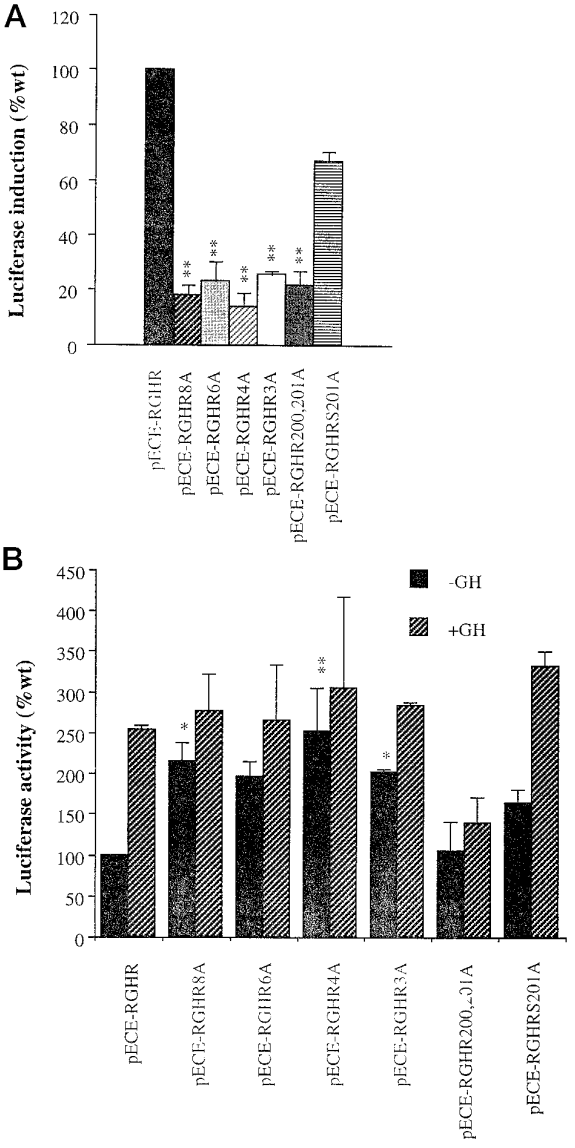

FIG. 1. *A*, comparison of alanine mutants and wild type RGHR in the induction of luciferase activity by GH. 1 μg/well of wild type RGHR or alanine mutant plasmids with 2 μg/well c-*fos* reporter plasmids were co-transfected into CHO K1 cells in six-well plates. After a 40-h incubation in Ham's F-12 medium containing 0.5% FCS, the cells were treated with 200 ng/ml GH for 5.5 h as described under "Materials and Methods." Data are means ± S.E. of three or four independent transfection experiments, and are expressed as a percentage of wild type RGHR induction. **, $p < 0.01$, by ANOVA with Tukey's multiple range comparison. *B*, comparison of basal luciferase activity of wild type RGHR and alanine mutants. All values of basal luciferase activity of alanine mutants were normalized to a basal luciferase activity of 100% for wild type RGHR. GH inductions for wild type RGHR and alanine mutants were similarly normalized. Data are means ± S.E. *, $p < 0.05$; **, $p < 0.01$ by ANOVA with Tukey's multiple range test.

278% of wild type (Table II). However, transient transfection assay showed that basal luciferase activities were similar to that of wild type RGHR with all of these cysteine mutants, ranging from 79% (pECE-RGHRS145C) to 115% (pECE-RGHRS201C) of wild type (Fig. 2*B*). Cysteine substitution of Ser-145 almost abolished GH induction of luciferase, exhibiting just 5.3 ± 2.9% (mean ± S.E.) the activity of wild type RGHR (Fig. 4). GH induction with pECE-RGHR-D152C, pECE-RGHR-Y200C, pECE-RGHR-S201C, and pECE-RGHR-152,200C was 12 ± 3%, 42 ± 5%, 64 ± 10%, and 10 ± 2.0% that of wild type RGHR, respectively (Fig. 2*A*). Accordingly, these substitutions further support the important role of dimerization domain residues (particularly Ser-145 and Asp-152) in signal transduction, but provide no evidence of constitutive activation of the receptor.

TABLE I
Salt bridges and hydrogen bonds across the dimerization domain (4)

hGHBP I atom	hGHBP II atom	Distance
		Å
$Ser^{145}O\gamma$	$Asp^{152}O\delta2$	3.0
$Leu^{146}N$	$Ser^{201}O\gamma$	3.1
$Thr^{147}O\gamma$	$Asp^{152}O\delta1$	2.7
$His^{150}N\epsilon2$	$Asn^{143}O\delta1$	2.9
$Asp^{152}O\delta2$	$Tyr^{200}O\eta$	2.7
$Ser^{201}O\gamma$	$Tyr^{200}O\eta$	3.3

TABLE II
Binding affinity and number of wild type and mutated RGHR

Constructs	Binding affinity	No. of RGHR/cell	No. of wild type RGHR
	$\times 10^{-10}\ M^{-1}$		%
pECE-RGHR	1.50 ± 0.3	3485 ± 63	100
pECE-RGHRN143K	1.37 ± 0.176	2544 ± 48	73
pECE-RGHRS145C	0.72 ± 0.09	9696 ± 3677	278
pECE-RGHRS145K	0.59 ± 0.11	5148 ± 1260	148
pECE-RGHRL146D	1.43 ± 0.6	1680 ± 529	48
pECE-RGHRL146K	1.31 ± 0.2	6048 ± 1660	173
pECE-RGHRT147K	1.24 ± 0.38	2640 ± 629	76
pECE-RGHRH150D	1.08 ± 0.13	2088 ± 615	60
pECE-RGHRD152C	1.48 ± 0.09	2640 ± 635	76
pECE-RGHRD152K	1.58 ± 0.02	1872 ± 680	54
pECE-RGHRY200C	1.30 ± 0.1	3936 ± 584	113
pECE-RGHRY200D	1.24 ± 0.26	1320 ± 433	38
pECE-RGHRS201A	1.50 ± 0.4	3024 ± 144	87
pECE-RGHRS201C	1.05 ± 0.28	3864 ± 536	111
pECE-RGHRS201D	0.98 ± 0.17	2790 ± 641	80
pECE-RGHRS201K	1.30 ± 0.3	1920 ± 584	55
pECE-RGHR150,152K	0.88 ± 0.14	1872 ± 72	54
pECE-RGHRH150D plus pECE-RGHRD152K	1.13 ± 0.18	1656 ± 288	48
pECE-RGHRS201D plus pECE-RGHRS201K	1.24 ± 0.17	3600 ± 831	103
pECE-RGHRY200D plus pECE-RGHR150,152K	1.40 ± 0.1	864 ± 288	25
pECE-RGHR8A	1.30 ± 0.1	986 ± 561	28
pECE-RGHR6A	1.41 ± 0.3	2160 ± 216	62

Aspartate Substitutions—Charge reversal is a useful tool for defining interactive residues (20–22). Conversion of residues in the dimerization region of RGHR to aspartic acid should abrogate dimerization by repulsion unless divalent cations shield these negative charges (21). Accordingly, four dimerization domain residues (leucine 146, histidine 150, tyrosine 200, and serine 201) were converted to aspartic acid. Scatchard analysis of stable CHO K1 populations expressing these mutant receptors showed that hormone affinity was not significantly different from wild type RGHR, and cell surface receptor expression ranged from 38–80% of wild type (Table II). Inductions of luciferase activity by GH with pECE-RGHR-L146D, pECE-RGHR-H150D, pECE-RGHR-Y200D, and pECE-RGHR-S201D were 90 ± 5%, 11 ± 3%, 27 ± 3.3%, and 13 ± 4% that of wild type RGHR, respectively (Fig. 3*A*). Wild type RGHR and the four aspartic acid mutants had similar basal luciferase activity (Fig. 3*B*). These data support a role for His-150, Tyr-200, and Ser-201 in the dimerization process, but not Leu-146.

Lysine Conversions—As in the case of aspartic acid, substitution of dimerization domain residues with lysine would be expected to inhibit dimerization by charge repulsion. Accordingly, 8 residues (asparagine 143, serine 145, leucine 146, threonine 147, histidine 150, aspartate 152, tyrosine 200, and serine 201) were converted to lysine. With the exception of Ser-145, hormone affinity did not differ from wild type in stable transfectants (Table II). As in the case of the cysteine mutant

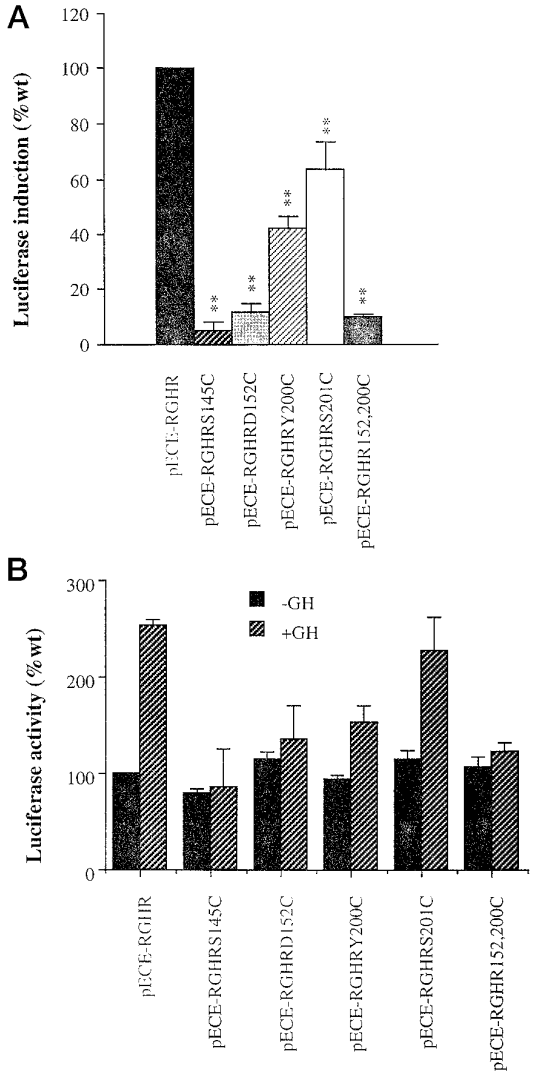

FIG. 2. *A*, comparison of cysteine mutants and wild type RGHR in the induction of luciferase activity by GH. Protocol and analysis were as described in Fig. 1*A*. *B*, comparison of basal luciferase activity of wild type RGHR and cysteine mutants. Analysis was as described in Fig. 1*B*.

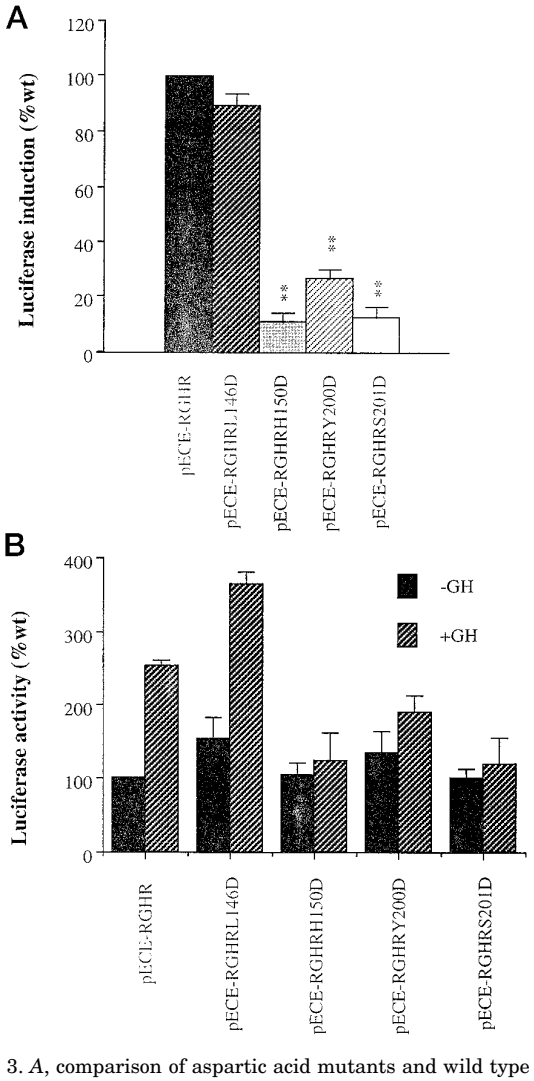

FIG. 3. *A*, comparison of aspartic acid mutants and wild type RGHR in the induction of luciferase activity by GH. Protocol and analysis were as described in Fig. 1*A*. *B*, comparison of basal luciferase activity of wild type RGHR and aspartic acid mutants. Analysis was as described in Fig. 1*B*.

RGHR-S145C, the binding affinity of RGHR-S145K was somewhat lower than that of wild type RGHR (39%), which may indicate a minor contribution to hormone binding, presumably indirectly through the β sandwich. Receptor expression in these stable populations ranged from 54 to 173% of wild type (Table II). Fig. 4*A* shows that pECE-RGHR-L146K and pECE-RGHR-T147K gave essentially wild type induction of luciferase activity by GH and pECE-RGHR-N143K showed minor loss of inducibility, while pECE-RGHR-S145K, pECE-RGHR-H150K, pECE-RGHR-D152K, pECE-RGHR-S201K, pECE-RGHR-150,152K, and pECE-RGHR-200,201K showed clearly reduced induction, particularly the last three mutants. This indicates that substitution of asparagine 143, leucine 146, and threonine 147 with lysine has little effect on signal transduction to c-*fos*, whereas again Ser-145, His-150, Asp-152, and Tyr-200/Ser-201 are needed for effective signaling. Interestingly, pECE-RGHRN143K displayed a significantly higher basal luciferase activity (264 ± 4% of wild type RGHR), although basal expression of other lysine mutants was normal (Fig. 4*B*).

Combination of Aspartate and Lysine Conversions—Taken together, these alanine, cysteine, aspartic acid, and lysine mutations indicate that particular dimerization domain residues are involved in signal transduction by the RGHR. However, the decrease in induction of luciferase activity by GH with these mutants could also result from a less specific perturbation in conformation in the C-terminal domain of the RGHR extracellular region, which affects signal transduction but not binding affinity. Therefore, to verify whether these residues are directly involved in dimerization, and to gain information about proximities of important dimerization domain residues, charge complementation studies (20) were undertaken. Plasmids encoding negatively charged mutants (pECE-RGHR-H150D, pECE-RGHR-Y200D, and pECE-RGHR-S201D) and positively charged mutants (pECE-RGHR-S145K, pECE-RGHR-L146K, pECE-RGHR-H150K, pECE-RGHR-D152K, pECE-RGHR-S201K, pECE-RGHR-150,152K, and pECE-RGHR-200,201K) were either transfected alone or co-transfected into CHO K1 cells with the c-*fos* reporter plasmid. The total amount of receptor expression plasmid was held constant, so that in the absence of complementation, a value between the individual values for single charge conversions would be predicted. Complementation of induction through electrostatic pairing would suggest that the decreased signaling observed when each mutant is expressed alone is specifically a result of disrupted dimerization.

Results with negatively charged mutants alone, and positively charged mutants alone or with both charge mutant com-

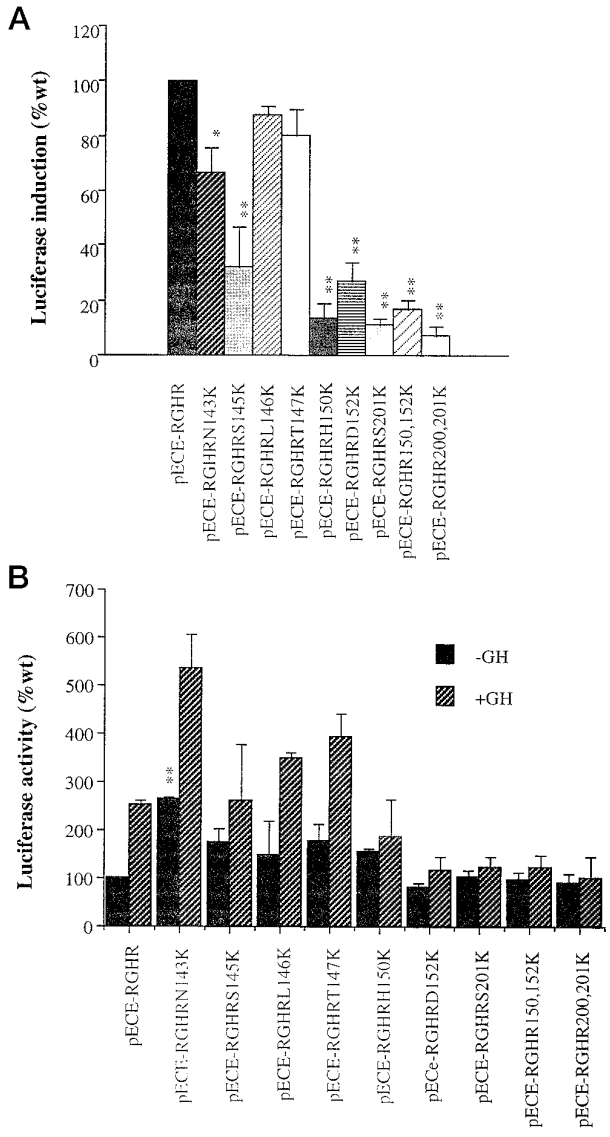

FIG. 4. *A*, comparison of lysine mutants and wild type RGHR in the induction of luciferase activity by GH. Protocol and analysis were as described in Fig. 1*A*. *B*, comparison of basal luciferase activity of wild type RGHR and lysine mutants. Analysis was as described in Fig. 1*B*.

TABLE III
Charge complementation studies

Constructs	Luciferase induction	Constructs	Luciferase induction
	%		%
RGHRwt	100	RGHRwt	100
H150D plus 150,152K	45 ± 5[a]	N143K plus H150D	62 ± 4
H150D	11 ± 3	N143K	67 ± 9
150,152K	24.5 ± 2	H150D	14 ± 7
H150D plus D152K	49 ± 7[a]	L146K plus S201D	39 ± 7
H150D	11 ± 3	L146K	88 ± 4
D152K	23 ± 8	S201D	10 ± 6
S201D plus S201K	33 ± 3[b]	H150K plus H150D	7 ± 6
S201D	17 ± 4	H150K	13.7 ± 6
S201K	10 ± 2	H150D	11 ± 3
Y200D plus 150,152K	40 ± 3[a]	200,201K plus Y200D	30 ± 4
Y200D	25 ± 2	200,201K	10 ± 4
150,152K	8 ± 6	Y200D	32 ± 5
Y200D plus D152K	39 ± 3[a]	S201K plus Y200D	17 ± 2
Y200D	25 ± 2	S201K	11 ± 2
D152K	21 ± 3	Y200D	20 ± 2

[a] $p < 0.05$, values are mean ± S.E.
[b] $p < 0.01$, values are mean ± S.E.

binations are summarized in Table III. These results showed that signal transduction was partially restored ($p < 0.05$ to $p < 0.01$) by charge complementarity between the 150/152 couple ($p < 0.05$), the 201/201 couple ($p < 0.01$), and the 200/150–152 couple ($p < 0.05$). Weak complementation probably also occurs between the 143/150 couple and the 200/201 couple, but not among the remaining charge complementations. Since these conclusions are based on the assumption that co-expressed plasmids are expressed as the sum of each separately, we tested this by co-transfecting three positive complementations and measuring total cell surface expression by Scatchard analysis. Table II shows that for the 150/152 couple and the 201/201 couple, receptor expression resulting from co-transfection is not significantly different from the sum of separate expressions. However, expression of cotransfected pECE-RGHR-Y200D and pECE-RGHR-150,152K is somewhat less than either construct alone; the effect of this will be to minimize the observed complementation.

DISCUSSION

It has been demonstrated that GH binding leads to dimerization and receptor signaling (6), but with the exception of a case of Laron dwarfism involving Asp-152 (9), the importance of individual residues within the dimerization domain has not been demonstrated. Indeed, to our knowledge, a mutagenic analysis of known receptor dimerization domain residues needed for signaling has not been carried out with any cytokine receptor, although a study on four putative dimerization domain residues has been reported for the erythropoietin receptor (19). Here we have shown that Ser-145, His-150, Asp-152, Tyr-200, and Ser-201 constitute the important residues required for effective signal transduction to the c-*fos* promoter, which integrates two of the major signaling pathways originating from the activated receptor: STAT (signal transducer and activator of transcription) and mitogen-activated protein kinases (11). We have also used a charge complementation approach derived from Barnard (20) to demonstrate that the loss in signaling seen with mutated residues is not a result of nonspecific conformational perturbation, and to demonstrate proximity of certain of the dimerization domain residues involved in signal transduction. Charge reversals have been used successfully in the study of a number of protein-protein interactions (*e.g.* Refs. 21 and 22).

Since the sequences of the rabbit and human GH receptors are totally homologous within the dimerization domain (13), in the ensuing discussion of individual interactions we shall refer to the distances determined for the human $(GHBP)_2$ hGH complex (4).

In the crystal structure, the distance between the γ oxygen atom of serine 201 (receptor 1 or bp 1) and the η oxygen atom of tyrosine 200 (bp 2) is 3.3 Å, and the distance between the γ oxygen atom of serine 201 (bp 2) and the nitrogen atom of leucine 146 (bp 1) is 3.1 Å (Table I), indicating hydrogen bonding. When serine 201 was converted to alanine, cysteine, aspartic acid, or lysine, induction of luciferase activity by GH with these mutants was 67%, 64%, 17%, and 11% that of wild type RGHR, respectively, and induction of luciferase activity by GH with pECE-RGHR-S201D and pECE-RGHR-S201K combined was 33% that of wild type RGHR. These results show that induction of luciferase activity by GH is greater when there is no repulsion between two position 201 residues, which means that proximity of both 201 residues is required for effective signal transduction by the RGHR, and indeed, these two residues are within salt bridge distance in the crystal structure. Charge complementation did not fully restore signaling, possibly because of steric hindrance in the dimerization

region or because not all transiently co-transfected cells expressed an equal amount of both constructs. The fact that signaling with S201C was not constitutive may mean that an additional hormone-induced conformational change is required, or that the disulfide bond did not form. Western blots of cysteine-substituted stable CHO K1 populations obtained from non-reduced, *N*-ethylmaleimide-extracted cells did not show receptor dimer, but did show a large amount of immunoreactive material at the stacking gel interface.[3]

In the crystal structure of the complex the η oxygen atom of tyrosine 200 (bp 2) is 2.7 Å from the δ2 oxygen atom of aspartic acid 152 (bp 1) and 3.3 Å from the γ oxygen atom of serine 201 (bp 1), and it is believed that tyrosine 200 forms an important hydrogen bond stabilizing the receptor dimer (Table I and De Vos *et al.* (4)). When tyrosine 200 was converted to cysteine and aspartic acid, the induction of luciferase activity by GH were 42% and 24% that of wild type RGHR, respectively, without significant change in hormone binding affinity. Furthermore, induction of luciferase activity by GH with pECE-RGHR-S201A and pECE-RGHR-200,201A was 67% and 21% that of wild type RGHR, respectively. These results verify that tyrosine 200 is important for signal transduction by the RGHR but has no significant effect on hormone binding affinity, as expected. In the case of Y200C, where the phenolic ring of tyrosine was replaced by a thiol group, conformational perturbation or weakening of the hydrogen bond is expected, with minor loss in signaling. However, in the case of Y200D, the mutant resulted in repulsion between two aspartic acids, so induction of luciferase activity by GH with pECE-RGHR-Y200D was lower than that of pECE-RGHR-Y200C, even accounting for decreased expression of the aspartate mutant (Table II). Further evidence for a role of tyrosine 200 in dimerization leading to signaling came from the charge reversals. Combinations of pECE-RGHR-150,152K with pECE-RGHR-Y200D and pECE-RGHR-D152K with pECE-RGHR-Y200D led to a greater induction of luciferase activity by GH than with RGHR-150,152K, pECE-RGHR-Y200D, and pECE-RGHR-D152K alone (Table III). Interestingly, pECE-RGHR-200,201K with pECE-RGHR-Y200D and pECE-RGHR-S201K with pECE-RGHR-Y200D did not give a greater induction of luciferase activity by GH than pECE-RGHR-200,201K, pECE-RGHR-Y200D, and pECE-RGHR-S201K alone, respectively (Fig. 5). These results suggested that tyrosine 200 interacts with aspartic acid 152 rather than serine 201. This suggestion was also supported by the minimal GH induction seen with the non-repulsive mutant pECE-RGHR-152,200C (Fig. 2). It may be that the inability to obtain constitutive activation with the pECE-RGHR152,200C mutant is a result of an excessive distance between thiol groups where disulfide bonds cannot be formed when the rigid β sheet structures are aligned, or a result of formation of non-signaling higher order polymers, as indicated above.

When aspartic acid 152 was converted to cysteine and to lysine, induction of luciferase activity by GH was 12% and 23% that of wild type RGHR, respectively, although hormone binding affinity and expression were similar to wild type RGHR (Table II). These results indicate that aspartic acid 152 is involved in signal transduction of RGHR and are consistent with a recent report that Laron dwarfism occurs when aspartic acid 152 is replaced by histidine (9). Combination of pECE-RGHR-150,152K with pECE-RGHR-H150D and of pECE-RGHR-D152K with pECE-RGHR-H150D resulted in complementation of luciferase induction by GH relative to pECE-RGHR-150,152K, pECE-RGHR-D152K, and pECE-RGHR-H150D alone, which indicates that interaction between

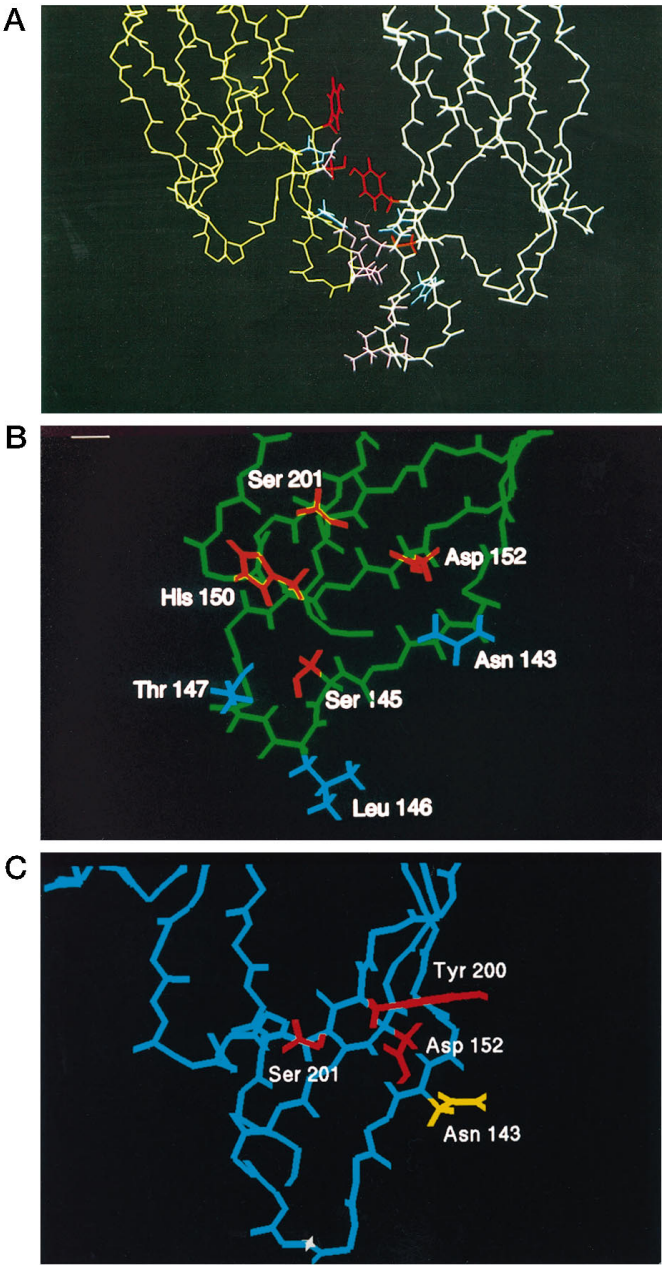

FIG. 5. **Receptor dimerization domains.** *A*, viewed from side. α carbon chains of the two receptor molecules are shown as *yellow* (bp 1) and *white* (bp 2), respectively. Based on the crystal structure (Table I), residues located in the dimerization domain are shown as *red* (Tyr-200, Ser-201), *blue* (His-150, Asp-152), and *pink* (Asn-143, Ser-145, Leu-146, and Thr-147) at the base of domains 2. *B*, face-on view of BP 1 dimerization domain, showing residues needed for signaling in *red*, and those not involved in *blue*. *C*, face-on view of BP 2 dimerization domain showing residues needed for signaling in *red*, and those not involved in *yellow*.

residues 150 and 152 is required for signal transduction. Indeed, in the wild type RGHR, the ϵ1 nitrogen atom of histidine 150 (bp 1) is 3.8 Å from the δ1 oxygen atom of aspartic acid 152 (bp 2), forming a weak salt bridge.

In the crystal structure, the distance between the ϵ2 nitrogen atom of histidine 150 (bp 1) and the δ oxygen atom of asparagine 143 (bp 2) is 2.9 Å, which suggests a hydrogen bond between them (4). Surprisingly, when histidine 150 was converted to either aspartic acid or lysine, signaling was reduced to less than 20% of wild type RGHR (Figs. 3 and 4), indicating the undesirability of a full charge at this location. Examination of the crystal structure shows that this is probably a result of

[3] M. J. Waters, unpublished observation.

the close proximity of Asp-152 on the one hand, and steric hindrance to the introduction of lysine on the other. Interestingly, when nearby asparagine 143 was converted to lysine, induction of c-*fos* promoter by GH was 67% that of wild type RGHR, while the basal luciferase activity was 265% that of wild type RGHR. Indeed, the small decrease of inducibility by GH seen with this mutant may be a result of higher basal luciferase activity rather than decreased dimerization of RGHR. Thus, constitutive signaling by RGHR appears to occur with this mutant. Coexpression of pECE-RGHRH150D and pECE-RGHRN143K showed lower GH induction of luciferase activity than pECE-RGHRN143K alone (despite similar receptor number), indicating that a salt bridge between aspartic acid 150 and lysine 143 is not forming due to repulsion by Asp-152. Examination of the crystal structure shows that lysine at 143 (bp 2) is within optimum H bond distance (3 Å) from Thr-147 (bp 1), whereas with asparagine at 143, the distance to threonine 147 is too great (6.4 Å). Thus, in the Lys-143 mutant, a new strong hydrogen bond forms, which may account for the apparent constitutive activity.

In the model of DeVos *et al.* (4), the distance between the γ oxygen atom of threonine 147 (bp 1) and the δ1 oxygen atom of aspartic acid 152 (bp 2) is 2.7 Å, and the distance between the nitrogen atom of leucine 146 (bp 1) and the γ oxygen atom of serine 201 (bp 2) is 3.1 Å. When threonine 147 was converted to lysine, induction of luciferase activity by GH with this mutant was 80% that of wild type RGHR and the binding affinity of this lysine mutant was 83% that of wild type RGHR, *i.e.* no significant effect was observed. Similarly, when leucine 146 was converted to lysine and to aspartic acid, induction of luciferase activity by GH with these mutants were 88% and 89% that of wild type RGHR, and binding affinity of these mutants were 87% and 95% that of RGHR, respectively. In addition, the combination of pECE-RGHRS201D with pECE-RGHRL146K exhibited a lower induction of luciferase activity by GH than pECE-RGHRL146K alone, suggesting little interaction (Table III). These results indicate that leucine 146 and threonine 147 are not involved in signal transduction by RGHR.

There may also be an interaction between serine 145 (bp 1) and aspartic acid 152 (bp 2), since the distance between them is 3 Å, according to the crystal structure (Table I). Accordingly, when serine 145 was converted to cysteine or lysine, induction of luciferase activity by GH with these mutants was 5% and 32%, respectively, and the binding affinity of these mutants were 48% and 39% of wild type, respectively. These results indicate that serine 145 is involved in dimerization and also has a minor influence on affinity for hormone, possibly through perturbation of loop 5 and 6 interactions with the hormone (4).

In summary, our results suggest that signal transduction by RGHR, presumably as a result of dimerization, requires interaction between residues asparagine 143 and histidine 150, between histidine 150 and aspartate 152, between aspartate 152 and tyrosine 200, and between serine 201 and serine 201. The distribution of these residues is shown in Fig. 5, where it can be seen the important residues form two small pockets, of area less than 60 Å^2. Despite the predominance of apolar residues in the dimerization domain (4), it can be seen from this figure that there is a cluster of hydrogen bonding/charge residues at the functional center of this domain.

Finally, while the results of the initial alanine scan support the individual charge and cysteine substitutions discussed above, pECE-RGHR-8A, pECE-RGHR-6A, pECE-RGHR-4A, and pECE-RGHR-3A exhibited a higher basal level of luciferase activity than wild type RGHR. Indeed, it is difficult to determine if the decrease in induction of c-*fos* promoter by GH seen with these mutants results from high basal activity as a result of enhanced dimerization or from elimination of dimerization. It is certainly possible that multiple alanine substitutions create a hydrophobic surface facilitating hydrophobic interactions through the methyl side chains, somewhat analogous to a leucine zipper. Consideration of Fig. 5 indicates that such a hydrophobic pocket is likely to occur on alanine substitution, and calculation (23, 24) of the binding free energy for a 100-Å^2 buried hydrophobic area (well within the available surface area shown in Fig. 5) yields a value of 10–11 kJ/mol, close to the value for the hormone-$(GHBP)_2$ complex (25). We attempted to determine if the increased basal luciferase was a result of constitutive dimerization by performing the post transfection and hormone treatment in the presence of 12.5 nM monoclonal antibody 5, which is known to block receptor dimerization (5). While this was effective in blocking signaling through the wild type receptor, it was without effect on either the basal activity of pECE-RGHR-8A or its residual GH induction, presumably because the epitope for monoclonal antibody 5 was destroyed, as demonstrated for the D152H Laron mutation (9).[4]

In relation to the creation of constitutively active receptors, it should be noted that this occurs both spontaneously with the erythropoietin receptor R129C erythroleukemia mutation (18) and can be induced by substituting cysteines at positions 132 and 132 within the putative dimerization domain of this homodimerizing cytokine receptor (19). However, in the latter case, the proportion of disulfide-bonded dimers was quite small, and no indication of the extent of higher polymers was given. As indicated, these prevented us from definitely concluding we had created disulfide-bonded GH receptor dimers. It is interesting that the same residues are able to form disulfide dimers in the erythropoietin receptor (*e.g.* residues 129), whereas our complementation data suggest that different residues interact across the dimerization domain, except for Ser-201. Also, alanine substitution of four putative dimerization domain residues with the erythropoietin receptor did not affect proliferative signaling (19), whereas in our case, blockade of GH induction was observed. Clearly there are major differences between these homologous receptors. These differences emphasize the lack of homology that exists between potential dimerization domain sequences (26). This would be an advantage in the design of specific small molecule antagonists capable of inhibiting dimerization, hence signaling. The studies presented here, which demonstrate that signaling can be inhibited by modifying 5 dimerization domain residues (Ser-145, His-150, Asp-152, Tyr-200, and Ser-201) in a tight cluster (Fig. 5), provide a target for rational design of such antagonists. In principle, this approach could find application in the creation of other cytokine receptor antagonists for use in the treatment of a variety of cancers.

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The Role of Receptor Dimerization Domain Residues in Growth Hormone Signaling

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